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Virulence-Associated Genetic Regions Comprising 31 Kilobases of the 230-Kilobase Plasmid in *Shigella flexneri* 2a

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By random transposon Tn*5* insertions, we previously identified six virulence-associated *Sal*I fragments, B, D, F, G, H, and P, in the 230-kilobase plasmid pMYSH6000 of *Shigella flexneri* 2a. In this study, we analyzed the sites of 134 independent Tn*5* insertions on four contiguous *Sal*I fragments, B, P, H, and D, of pMYSH6000 and identified five virulence-associated regions; four were associated with inducing a positive Sereny test (Ser), invasion into epithelial cells (Inv), binding to Congo red (Pcr), and inhibition of bacterial growth (Igr), and one was associated with the Ser and Inv but not with the Pcr or Igr phenotypes. Hybridization studies revealed that these virulence-associated DNA regions were highly conserved among 15 other virulence plasmids of four species of *Shigella* and enteroinvasive *Escherichia coli*. These data indicate that at least seven separate genetic determinants on the virulence plasmid are required for full expression of the virulence phenotype of shigellae.

Shigellae are enteroinvasive bacteria that cause bacillary dysentery in humans and monkeys. These organisms invade colonic epithelial cells, multiply intracellularly, and spread to adjacent cells (9). The genetic determinants required for these abilities are located on at least three separate sites of the chromosome (3–5, 18) and on a 100- to 140-megadalton (MDa) plasmid. Commonly, plasmids of shigellae and enteroinvasive *Escherichia coli* (EIEC) contain genetic regions that are required in the early steps of the invasion process (18, 19); loss of the plasmid or deletion of an essential region consistently leads to loss of virulence (13, 21).

Expression of virulence in shigellae is dependent on temperature (12). Shigellae grown at 37°C are fully virulent, whereas bacteria grown at 30°C neither invade epithelial cells (12) nor provoke keratoconjunctivitis in guinea pigs (24). Making use of this property, Hale et al. (6) identified at least seven plasmid-coded, virulence-associated peptides produced by *Shigella flexneri* 2a and 5 and EIEC strain O143. By Western blot (immunoblot) analysis of extracts of whole cells, four peptides of 78, 62, 43, and 38 kDa were recognized by convalescent-phase monkey antisera. These workers proposed that these proteins function as components of the invasion phenotype and are expressed on the bacterial surface. Oaks et al. (15) identified an additional plasmid-encoded surface peptide of 140 kDa which was also specifically recognized by convalescent-phase human or monkey sera. To identify the genetic regions associated with invasion, Maurelli et al. (14) shotgun-cloned *Sau*3A digests of the plasmid DNA into a cosmid vector, which was subsequently introduced into plasmid-free *S. flexneri* 5. A clone containing a 37-kilobase (kb) minimum sequence necessary for invasion was isolated. This recombinant clone also produced the four virulence-associated peptides described by Hale et al. (6). A DNA fragment coding for three antigenic proteins of 57, 43, and 39 kDa was cloned into a λ expression vector by Buysse et al. (1). The genetic determinants for virulence-associated proteins were localized on a 9-kb DNA region of the cloned fragment. These studies suggested that eight to nine proteins encoded by the large plasmid play a role in virulence.

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In an earlier study, we reported on the *Sal*I restriction map of the 230-kb plasmid, pMYSH6000, of *S. flexneri* 2a YSH6000 (21). By random transposon Tn*5* insertion mutagenesis, we have shown that insertions in *Sal*I fragments F and G and insertions in the contiguous fragments B, P, H, and D resulted in the avirulent phenotype (22). Insertions in *Sal*I-G induced the loss of the Ser phenotype (Sereny test) but not the Inv (invasion), Pcr (binding to Congo red), or Igr (inhibition of bacterial growth) phenotypes (that is, Ser^- Inv^+ Pcr^+ Igr^+) (10, 22). The locus affected was localized within a 4.5-kb region tentatively designated *virG* (10). Insertional mutations in *Sal*I-F were localized within a 1-kb *virF* gene (16, 17) and resulted in a Ser^- Inv^- Pcr^- Igr^- phenotype.

In this communication we extend our studies to the contiguous B-P-H-D region. Tn*5* insertional mutagenesis defined four regions that were associated with Ser^+ Inv^+ Pcr^+ Igr^+ virulence phenotypes and a fifth region that was associated with Ser^+ and Inv^+ but not Pcr^+ or Igr^+. By Southern hybridization studies, we have also shown that the B-P-H-D region is highly conserved among several species of shigellae as well as EIEC.

MATERIALS AND METHODS

Bacterial strains. *S. flexneri* 2a YSH6000 (21) was used for identifying the virulence-associated regions in the 230-kb plasmid pMYSH6000 (21, 22). Three different isolates of each of the following strains, *S. dysenteriae*, *S. flexneri*, *S. boydii*, *S. sonnei*, and EIEC, were used for extracting plasmid DNA and for testing sequence homology with eight contiguous *Eco*RI fragments that cover the 31-kb virulence-associated region of pMYSH6000.

Media and chemicals. Organisms were cultured in LN (20) or Trypticase soy (BBL Microbiology Systems, Cockeysville, Md.) broth in liquid or in medium solidified with agar. Congo red was purchased from Sigma Chemical Co., St. Louis, Mo.

Insertion mutagenesis of pMYSH6000. Random insertions of Tn*5* were generated in pMYSH6000 by use of the replication-thermosensitive transposon delivery vector pCHR81 [R388Rep(Ts)::Tn*5*] (23) as described previously (22).

Isolation and characterization of plasmid DNA. Large- and

small-scale preparation of plasmid DNA has been previously described (22). Restriction endonucleases were used as described by Maniatis et al. (11). Electrophoresis of restriction endonuclease-cleaved DNA was performed in horizontal 0.9 or 0.7% agarose slab gels in a Tris-acetate buffer system (22).

Determination of Tn*5* insertion sites in pMYSH6000. Sites of Tn*5* insertion were assigned to each *Sal*I and *Eco*RI fragment of pMYSH6000. Each *Sal*I fragment with Tn*5* was shotgun-cloned to pBR322 by selecting for kanamycin resistance. Since Tn*5* has only one *Sal*I site in its middle part and outside of the Km^r gene, the *Sal*I-cleaved Km^r clones obtained consist of the 2.7-kb Km^r segment of Tn*5*, a part of the *Sal*I fragment of pMYSH6000, and pBR322. Digestion of these clones with *Sal*I and with *Sal*I and *Eco*RI enabled us to estimate the distance between the site of Tn*5* insertions and either the *Sal*I or *Eco*RI site nearest to it. If the size of the *Sal*I or *Eco*RI fragment to be measured was larger than about 8 kb, *Hin*dIII or *Bgl*II was used instead of *Eco*RI to obtain a fragment that was smaller than 8 kb so that the measurement was more accurate. In this manner, sites of Tn*5* insertion on pMYSH6000 could be determined with an error of less than 200 base pairs (bp).

DNA-DNA hybridization. Hybridization on nitrocellulose filters was performed under stringent conditions by the Southern blot hybridization method (25).

Test for virulence-associated phenotypes of shigellae. The four virulence-associated phenotypes of each Tn*5* insertion mutant were assayed as described previously (22).

Plaque assay. LLC-MK2 cells (MK2) (21) were grown to confluency in 96-well microtiter plates (Nunc, Roskilde, Denmark) at 37°C in a humidified atmosphere of 5% CO_2. The medium in the wells was removed from MK2 cell monolayers and replaced with 250 μl of fresh antibiotic-free Eagle minimal essential medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 5 mM glutamine and 5% fetal bovine serum (General Scientific Laboratories, Los Angeles, Calif.). Bacterial strains grown at 37°C were diluted with saline to a concentration of approximately 5×10^8 cells per ml. After 50 μl of the bacterial suspension was added to each well, microtiter plates were incubated at 37°C in 5% CO_2 for 2 h. Each well was washed twice with Hanks balanced salt solution and filled with 300 μl of Eagle medium containing 100 μl of gentamicin per ml and incubated at 37°C in 5% CO_2 for an additional 1 h. The medium was changed, and the microtiter plates were kept at 37°C in 5% CO_2 for 72 h. The plaque-forming ability of each strain was scored at 24-h intervals under low magnification. YSH6000 and the 230-kb plasmid-free avirulent derivative, YSH6200 (16), were routinely used as the positive and negative controls, respectively, in each assay.

RESULTS

Invasion-associated genetic regions in the 230-kb virulence plasmid. Figure 1 shows the restriction map of the 31-kb contiguous *Sal*I fragments B, P, H, and D and the location of the Tn*5* insertions. Insertion mutations that resulted in the avirulent phenotype (Ser^- Inv^- Pcr^- Igr^-) appeared to reside in at least four distinctive regions, tentatively designated regions 1, 3, 4, and 5. The map positions of the various insertions are also shown in Fig. 1. Ten of 14 mutants with insertion mutations in *Sal*I-F showed similar phenotypes (Ser^- Inv^- Pcr^- Igr^-), and their insertion sites were local-

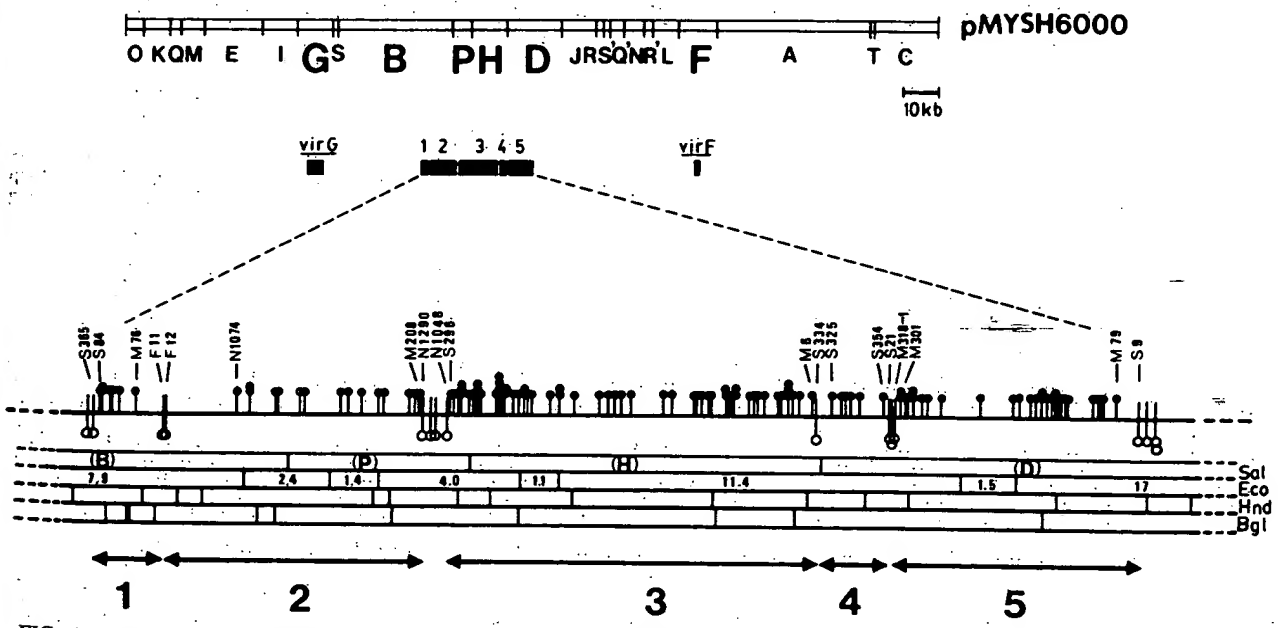

FIG. 1. Restriction endonuclease map and sites of Tn*5* insertion within the 31-kb virulence-associated genetic region of pMYSH6000. The *Sal*I restriction map of pMYSH6000 (21) is shown at the top. Below that, Tn*5* insertions (solid circles) that resulted in loss of the invasive phenotype (Inv^-) are indicated. The numbers above the circles represent the outermost sites of Tn*5* insertion for each virulence-associated region. The maximum size of each virulence-associated region in the 31-kb segment is shown at the bottom by arrows. Tn*5* insertions that resulted in the Inv^+ phenotype are also shown (open circles), and the minimum segment between each virulence-associated region is represented by numbers. Some of the independently generated Tn*5* insertions are at the same location. The black boxes beneath the *Sal*I map indicate the location of *virG*, regions 1, 2, 3, 4, and 5, and *virF* on the *Sal*I map. Restriction endonuclease maps with three additional enzymes, *Eco*RI (Eco), *Hin*dIII (Hnd), and *Bgl*II (Bgl), of the 31-kb virulence-associated regions are shown beneath the map of Tn*5* insertions. The numbers for each of the *Eco*RI restriction fragments are the size (in kilobases).

ized within a 1-kb region (data not shown). This region corresponded to the *virF* gene (16, 17), which was about 43 kb apart from the B-P-H-D cluster. Hence, five genetic regions were identified in which mutagenesis resulted in the loss of all four virulence-associated phenotypes.

In the initial phase of this study, we isolated an insertion mutant altered in *Sal*I-P which lost the Ser^+ Inv^+ phenotype but not the Pcr^+ Igr^+ phenotype (Ser^- Inv^- Pcr^+ Igr^+). This observation suggested that there was an additional genetic determinant between regions 1 and 3. By selecting for insertion mutants that were Pcr^+ but avirulent according to the plaque assay (see Materials and Methods), 15 insertional mutants from 1,500 random Tn*5* insertions were isolated with the Ser^- Inv^- Pcr^+ Igr^+ phenotype. All of these mutations mapped within a 5.1-kb cluster that straddled the junction between *Sal*I-B and -P. This region was tentatively designated region 2. As summarized in Table 1, region 2 seemed to be a new type of genetic determinant and to play a different role in invasion from that of *virF* and regions 1, 3, 4, and 5.

Conservation of the sequence homology of the 31-kb virulence-associated region. *Eco*RI fragments of the 31-kb virulence-associated region of pMYSH6000, which covers regions 1 through 5, were used to prepare DNA probes to examine DNA sequence homology between plasmids of various *Shigella* and EIEC strains. Plasmid DNA prepared from the various strains was digested with *Eco*RI and subjected to Southern hybridization with the eight ^{32}P-labeled probes (Fig. 2). Twelve of the strains had *Eco*RI fragments that were the same size as and homologous to the corresponding probe fragments from pMYSH6000 (Fig. 2A to G). One strain of *S. dysenteriae* and one strain of *S. boydii* lacked the 7.9- and 2.4-kb *Eco*RI fragments and instead had a 10.3-kb fragment which was homologous to both the 7.9- and 2.4-kb probes (Fig. 2A and B, lanes 3 and 9). This was very likely due to the loss of an *Eco*RI site between the 7.9- and 2.4-kb fragments. The same *S. dysenteriae* strain also had a 12.9-kb *Eco*RI fragment which hybridized to both the 11.4- and 1.5-kb probes (Fig. 2F and G, lane 3). This can also be ascribed to the loss of an *Eco*RI site between the 1.4- and 4.0-kb fragments (Fig. 3). When homology was probed with the 3.7-kb *Eco*RI-*Xho*I probe, which consists of one-third of region 5 on the rightmost side, various sizes of hybridizable *Eco*RI fragments were noted (Fig. 2H). It is of interest that all of these homologous fragments were considerably larger than 3.7 kb. These results suggest that the virulence-associated cluster itself is highly conserved. Molecular reconstruction may, however, occur outside this region. Perhaps the *Eco*RI site at the left site of the *Sal*I B-P-H-D cluster is conserved, because this region is close to the *virG* site.

TABLE 1. Phenotypes of mutations in each of the virulence-associated regions of pMYSH6000

Gene or region	Phenotype			
	Ser	Inv	Pcr	Igr
virG[a]	−	+	+	+
Region 1	−	−	−	−
Region 2	−	−	+	+
Region 3	−	−	−	−
Region 4	−	−	−	−
Region 5	−	−	−	−
virF	−	−	−	−

[a] *virG*, formerly designated VirG (10), has been confirmed to be a cistron (unpublished data).

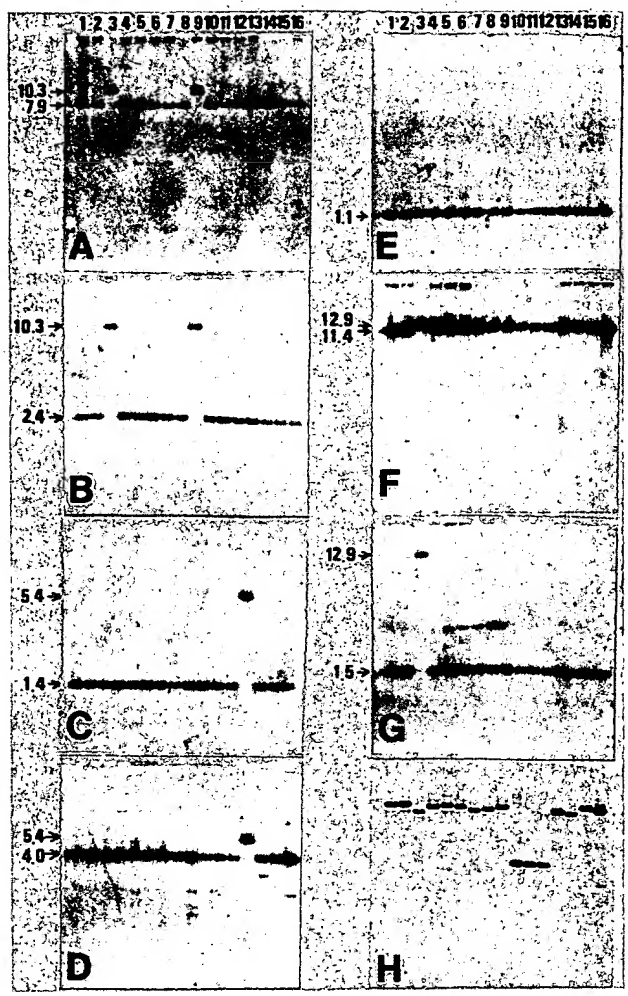

FIG. 2. Southern blot hybridization of *Eco*RI-cleaved virulence plasmids extracted from various shigellae and EIEC strains with DNA probes specific for eight contiguous *Eco*RI fragments covering the 31-kb virulence-associated region of pMYSH6000. The eight DNA probes were prepared from 7.9-, 2.4-, 1.4-, 4.0-, 1.1-, 11.4-, and 1.5-kb *Eco*RI fragments and the left 3.6-kb *Eco*RI-*Xho*I part of the 17-kb *Eco*RI fragment (see Fig. 1 and 3). Panels A through H correspond to DNA-DNA hybridizations with the eight DNA probes listed above, respectively. Lanes 1 through 16, Plasmids extracted from three different isolates each of *S. dysenteriae* (lanes 1 to 3), *S. flexneri* (lanes 4 to 6), *S. boydii* (lanes 7 to 9), *S. sonnei* (lanes 10 to 12), EIEC (lanes 13 to 15), and YSH6000 as the control (lane 16). The sizes of the *Eco*RI restriction fragments (in kilobases) that hybridized with the DNA probes used are indicated.

Hybridization studies were also carried out (unpublished data) with the 1-kb *virF* gene as the DNA probe. This gene, which is located as far as 43 kb apart from the virulence-associated cluster, hybridized to *Eco*RI fragments whose size varied among the different strains from 9 to 30 kb.

DISCUSSION

By random Tn*5* insertions, we have identified a total of seven virulence-associated regions on pMYSH6000; two were previously found on *Sal*I-G (10, 22) and *Sal*I-F (16, 17), and five were newly defined on a cluster of four *Sal*I fragments, B-P-H-D. The 4.5-kb *virG* gene on *Sal*I-G is

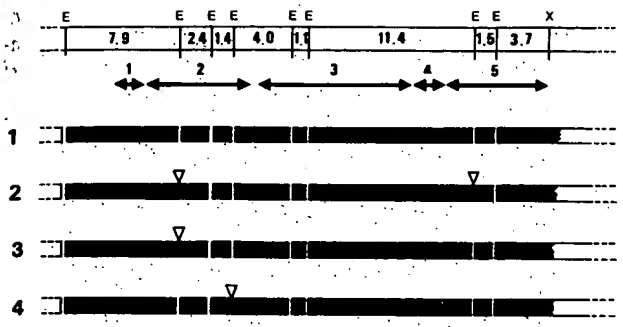

FIG. 3. Conservation of the 31-kb virulence-associated region of pMYSH6000 among 15 other different virulence plasmids. Thick black boxes indicate maps of the *Eco*RI fragments in the 15 virulence plasmids that are homologous to the corresponding probes of pMYSH6000 as shown at the top. Five segments, designated 1 through 5, correspond to the virulence-associated regions on the 31-kb cluster of pMYSH6000 (see Fig. 1). Open triangles indicate *Eco*RI cleavage sites presumably lost in each plasmid: type 2 (Fig. 2, lane 3) for one *S. dysenteriae*, type 3 (Fig. 2, lane 9) for one *S. boydii*, and type 4 (Fig. 2, lane 13) for one EIEC. All other plasmids belong to type 1. E, *Eco*RI; X, *Xho*I. Sizes are shown in kilobases.

located at 1.2 through 6.7 kb from the junction of *Sal*I-I and -G (10; unpublished data) and is required for a step immediately after invasion (10, 22). The genetic loci required for invasion are located on a cluster consisting of four *Sal*I fragments, B, P, H, and D, and on fragment F located 43 kb away from the terminus of the cluster (*Sal*I-D). *virF* is 1 kb long and is located 6.6 kb from the junction of *Sal*I-F and -L (16, 17). The distance from *virG* via the B-P-H-D cluster to *virF* is about 106 kb, almost half of the entire 230-kb-long plasmid. On the other hand, the distance between the outermost extremities of *virF* and the *Sal*I-B terminus of the clustered virulence regions on the fragment, B-P-H-D, is about 67 kb. Thus, the genetic loci required for invasion cannot be cloned as a single DNA fragment into a cosmid vector in the case of pMYSH6000. The remaining half of the plasmid consists of 124 kb and contains, near the middle, a region required for maintenance of the plasmid (*Sal*I-C; manuscript in preparation). All of these virulence- and replication-associated regions are necessary for shigellae and EIEC to be fully virulent. This positional relationship may permit maintenance of the virulence plasmid without marked internal change.

By analysis of Tn*5* insertions, the 31-kb virulence-associated B-P-H-D cluster of pMYSH6000 was divided into five regions. Tn*5* insertions in regions 1, 3, 4, or 5 resulted in the same avirulent phenotypes as Tn*5* insertions in the *virF* region (Ser^- Inv^- Pcr^- Igr^-). In contrast, region 2 must encode functions that differ from those encoded by regions 1, 3, 4, 5, *virF* (16, 17), and *virG* (10). Thus, region 2, when insertionally inactivated, loses the Ser^+ and Inv^+ abilities but not the Pcr^+ and Igr^+ phenotypes (Table 1). Therefore, region 2 appears to play a role in invasion at steps that are different from those of *virF* and of Regions 1, 3, 4, and 5.

The *Sal*I, *Eco*RI, and *Xho*I restriction patterns of the 31-kb region of pMYSH6000 were similar to those observed with a 37-kb DNA sequence of pWR100 of *S. flexneri* 5 (14). This region was cloned into a high-copy-number cosmid vector, pJB8 (pSH4108), by Maurelli et al. and shown to be a minimum DNA sequence necessary for *Shigella* invasion (14). Thus, these results differ somewhat from ours, since pSH4108 is able to induce invasion in the absence of the *virF* gene. However, it should be pointed out that the invasion phenotype induced by pSH4108 in *S. flexneri* and *E. coli* K-12 is not as efficient as the invasion phenotype induced by the parental invasion plasmid, pWR100 (14). Perhaps the phenotype observed in the absence of *virF* is a reflection of the high-copy-number effects of pJB8. Evidence has now been obtained which implicates *virF* as a positive regulator which affects the level of proteins produced by region 2 and *virG* (manuscript submitted). It may thus be that in our system the *virF* gene is required to induce the synthesis of proteins to a sufficiently high level so that invasion can proceed.

Buysse et al. (1) also cloned a 9-kb fragment that encodes three virulence-associated surface proteins of 57, 43, and 39 kb. The *Eco*RI cleavage pattern resembled that obtained with a part of pHS4108 (14); the *Eco*RI, *Hin*dIII, and *Bgl*II cleavage patterns corresponded to those of region 2 of the B-P-H-D cluster of pMYSH6000. Watanabe and Nakamura isolated a recombinant with a virulence-associated insert of 20 kb from a 120-MDa plasmid, pSS120, of *S. sonnei* (26). The *Hin*dIII, *Eco*RI, *Bgl*II, *Sal*I, and *Xho*I cleavage patterns of the fragment suggested that it was similar to region 5 of pMYSH6000. Daskaleros and Payne isolated a clone associated with the ability to bind Congo red from a virulence plasmid of *S. flexneri* 1b (2). Although the phenotype induced in *E. coli* K-12 by *virF* and the recombinant described by Daskaleros and Payne was similar, different restriction maps of the Pcr determinant were obtained.

The *Sal*I-defined B-P-H-D cluster is highly conserved in shigellae and EIEC and appears to play a central role in the invasion process. The requirement for multiple determinants for invasion by these organisms differ markedly from *Yersinia pseudotuberculosis*, which only requires a single outer membrane protein. We are currently undertaking studies to characterize the cistrons of the virulence-associated regions and their protein products in order to define their role in the pathogenesis of bacillary dysentery.

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